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Zonal release of proteins within tissue engineering scaffolds

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Abstract The manufacture of a scaffold for tissue engineering applications that can control the location and timing of growth factor release is described. The scaffold is formed by the sintering of poly(DL-lactic acid) ($P_{DL}LA$) microparticles, plasticized with poly(ethylene glycol) (PEG), although the method can be used for many other polymer types. The microparticles were loaded with model proteins, trypsin and horseradish peroxidase (HRP), or recombinant human bone morphogenetic protein-2 (rhBMP-2). Entrapment efficiencies above 75% were achieved using a solid-in-oil-in-water system. Controlled release of active protein was achieved for at least 30 days. Microparticles were built into protein-loaded or protein-free layers and release of the protein was restricted to zones within the scaffold. Cell response to rhBMP-2 was tuneable by changing the dose of the rhBMP-2 released by varying the ratio of protein-loaded and protein-free microparticles within scaffolds. Zonal activity of rhBMP-2 on C2C12 cells was demonstrated. The scaffolds may find utility in applications where gradients of growth factors within 3D templates are required or where zonation of tissue growth is required.

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Introduction

This paper describes a new scaffold that can release growth factors or other molecules from precise locations within its 3D structure. The concept of releasing growth factors over extended and controlled periods of time is widely pursued within the field of tissue engineering [1, 2]. Examples of approaches to control cell interactions with growth factors include encapsulation within a polymer scaffold and release following delayed hydration and/or polymer degradation [3], cell responsive release from hydrogels [4] and surface immobilization of growth factors [5]. The delivery of two different growth factor types from a scaffold has also been demonstrated to enhance tissue regeneration processes such as angiogenesis and bone regeneration [6].

The approach described in this paper provides a method of creating gradients of one or more growth factors and predetermining the location of release within the scaffold. We anticipate that such an approach can find utility in the use of scaffolds at tissue interfaces (e.g. osteochondral) or where growth factor gradients are required to direct cell movement or growth (e.g. nerve repair). A recent report of oligo(polyethylene glycol)fumarate) hydrogel scaffolds has proposed a similar strategy for osteochondral repair [7].

Our approach uses growth factor loaded microparticles that may be built layer-by-layer into a 3D structure. The loose aggregate of microparticles is then sintered to form an interconnected porous matrix. The growth factor loaded particles may be mixed with blank microparticles to tune the dosage and position of release. This paper details the technical development of the microparticle fabrication method to maximise protein entrapment, the formation of scaffolds and demonstration of location-specific release profiles using enzymes as model proteins. Finally, we demonstrate the location-specific control of osteogenic differentiation of

C2C12 cells within a scaffold containing recombinant bone morphogenetic protein-2 (rhBMP-2).

Materials and methods

Materials

Poly(D,L-lactide) ($P_{DL}LA$) was obtained from Purasorb (100 DL Low IV®, average molecular weight 66 kDa), poly vinyl alcohol (PVA) (MW = 25,000, 88% mol hydrolized, Aldrich), polyethylene glycol (PEG400 & PEG 18500) were purchased from Polyscience, Horseradish peroxidase (HRP) was purchased from Roche Diagnostic, Trypsin, N-benzoyl-DL-arginine-p-nitroanilide hydrochloride (BAPNA), 3,5,3′,5′-Tetramethylbenzidine (TMB) Liquid Substrate System Supersensitive Form for ELISA, p-nitrophenolphosphate (pNPP) substrate were all purchased from SIGMA. Recombinant human BMP-2 (rhBMP-2) was generously donated by Professor Walter Sebald (University of Wurzburg, Germany). Dulbecco's modified Eagle's medium (DMEM) and tissue culture reagents were obtained from Gibco/BRL (Scotland, UK). All other reagents were purchased from Sigma-Aldrich Co., Ltd. (Poole, UK), or BDH/Merck, Ltd. (Poole, UK) and were of reagent grade.

Methods

Production of protein-loaded microparticles—water-in-oil-in-water (w/o/w) double emulsion

Protein-loaded microparticles were fabricated using a w/o/w double emulsion technique [8]. In brief, 13.3 mg trypsin in 120 μL PBS was emulsified for 2 min using a vortex mixer (vortex V20, scale 5) with 1.5 mL of a polymer solution containing 0.5 g $P_{DL}LA$ and 0.15 g PEG400 in 1.5 mL dichloromethane, to form the primary emulsion. Subsequently, 3 mL of 0.3% (v/v) PVA was added and vortexed at scale 3 for 20 sec to form the double emulsion. The w/o/w emulsion was then poured into 50 ml 0.3% (v/v) PVA solution containing 5% (v/v) isopropanol and stirred continuously for 3 h until the solvent had evaporated and microparticles hardened. The hardened microparticles were filtered, washed 3 times with dH_2O, freeze dried and stored under dessicant at 4°C.

Production of protein-loaded microparticles—solid-in-oil-in-water (s/o/w) emulsion

Protein-loaded microparticles were also fabricated using a s/o/w emulsification/solvent evaporation technique using a modification of the method described by Morita et al. [9]. This method consisted of two consecutive-independent steps: (1) solid dispersion of protein and (2) emulsification/solvent evaporation. Solid dispersion of protein in PEG18500 was prepared by dissolving the protein (13.3 mg trypsin, 2 mg HRP, or 50 μg rhBMP-2) in 0.25 mL dH_2O and mixing with a solution of 0.015 g PEG18500 in 0.25 mL dH_2O. The solution was frozen in liquid nitrogen and freeze-dried for 48 h. The resulting solid was dissolved in dichloromethane and mixed with 1.5 mL polymer solution, containing 0.5 g $P_{DL}LA$ and 0.15 g PEG400 in 1.5 mL dichloromethane. The solution was emulsified with 3 mL 0.3% (v/v) aqueous PVA by vortexing at scale 3 for 20 sec followed by solvent evaporation as described above.

Determination of entrapment efficiency and retention activity of protein in microparticles

Microparticles with an equivalent weight to 2 mg of Trypsin or 0.3 mg of HRP were dissolved in 1.0 mL ethyl acetate. The solution was mixed with 2 ml PBS for 1 min using the vortex mixer, followed by centrifugation at 5°C, 5000 rpm for 5 min. The water phase was removed and the extraction procedure repeated using a further 2 mL and 1 mL of PBS. The water phase aliquots were pooled and the protein concentration determined using a micro-BCA protein assay. Enzyme activities of trypsin and HRP were analysed spectrophotometrically using a substrate BApNA and TMB liquid substrate system (Sigma, UK) respectively.

Scanning electron microscopy

Microparticles were sputter coated with gold for 4 min under an argon atmosphere in a Blazers SCD 030 sputter coater unit and the structure visualized using a Philips SEM 505 with Semicaps 2000A imaging system or a Jeol 6060LV SEM at an accelerating voltage of 25 kV and 12 kV respectively.

Differential scanning calorimetry

Various microparticle formulations containing PEG400 (0, 10, 20, and 30% $w/w/P_{DL}LA$) and 3% (w/w) PEG18500 and 30% (w/w) PEG400 / $P_{DL}LA$ with various water content were analysed using differential scanning calorimetry (DSC) to determine the glass transition temperature (Tg). Using a differential scanning calorimeter (Universal V2.5H TA instruments), scans of 10 mg samples were carried out from –40 to 120°C at a heating and cooling rate of 10°C /min under N_2 atmosphere. The Tg was evaluated as the midpoint of the inflection of the transition curve from the second heating scans (the initial scan was performed to eliminate the thermal history of each sample).

Moisture content

The moisture content was determined using an Ohaus MB45 moisture analyser. Samples of microparticles containing 3% (w/w) PEG18500 and 30% (w/w) PEG400 / $P_{DL}LA$ were analysed at various stages of the freeze drying process. Microparticle formulations (0.5 g) were dried in the analyzer at 102°C to a constant weight. The moisture content was expressed on a dry weight basis.

Scaffold fabrication

Scaffolds (10 mm height × 5 mm diameter) were prepared from microparticles using a heat sintering method (10). In brief, 100 mg of microparticles with a specific size range of 212–500 μm (collected following sieving) were poured into a 5 mm diameter well in a Teflon mould and heated at 70°C for 4 h. Following heating, the scaffolds were cooled to room temperature to terminate microparticle fusion, removed from the mould and stored in a dessicator at 4°C until required for further use. The morphology of the scaffolds was examined under SEM as described above.

Protein layered scaffolds were fabricated to demonstrate zonal release of proteins. For the release of HRP, a triple layer scaffold was made which consisted of a single layer of microparticles containing no HRP sandwiched between two layers of HRP loaded microparticles. The scaffold was prepared in a similar fashion as described above; 100 mg of HRP-loaded microparticles were transferred to the Teflon mould and the surface levelled off. Subsequently, 200 mg of non-protein loaded microparticles were placed on top followed by the final layer of HRP-loaded microparticles (10 mm height × 10 mm diameter).

For the release of rhBMP-2, a two layer rhBMP-2 scaffold was fabricated and consisted of a rhBMP-2 loaded zone and non rhBMP-2 zone using 50 mg rhBMP-2 loaded microparticles and 50 mg of non-protein loaded microparticles (10 mm height × 5 mm diameter). The loading of the rhBMP-2 zone was varied by using a mixture of rhBMP-2 loaded: non-rhBMP-2 loaded microparticles at ratios of 100:0 (100%), 50:50 (50%), 25:75 (25%) and 0:100 (0%) respectively.

Compressive strength

Compressive strength properties of the scaffolds were measured at room temperature using a Lloyd tensile testing machine (Model M30K) equipped with a 500 N load cell. The cylindrical scaffolds (10 mm height × 5 mm diameter) were compressed with a crosshead speed of 1.0 mm/min to measure the max compressive load per unit cross-sectional area.

Porosity

Geometrical porosity of the scaffold was calculated from the equation:

$$\text{Porosity } (\%) = (1 - \rho_{\text{scaffold}}/\rho_{\text{microparticles}}) * 100$$

ρ_{scaffold} = the density of the scaffold, which was measured geometrically

$\rho_{\text{microparticles}}$ = the density of the microparticles, which was measured using Helium pycnometry ($1.2253 \pm 0.0034 \text{g/cm}^3$)

Scaffold porosity was calculated from the average of 6 samples for each formulation.

In vitro protein release from scaffold and microparticles

Scaffold and microparticles with an equivalent weight to 0.3 mg of HRP were incubated in 1 ml of PBS (pH = 7.4) at 37°C and agitated on a plate shaker at 25 rpm. The samples were withdrawn at various intervals and the medium was replaced with the same amount of fresh medium. The concentration of protein released within samples was determined using a micro-BCA protein assay and the enzyme activity of HRP was analysed using TMB liquid substrate system (Sigma, UK).

Demonstration of location specific release HRP

Zonal release of proteins within scaffold was demonstrated using a three layer HRP scaffold. The microparticles were heat sintered as described above and the zonal release of HRP within the scaffold visualized by adding a few drops of TMB substrate to the scaffold. The sequential colour changes were recorded using a video camera from which stills were extracted.

Scaffold seeding and subsequent culture

C2C12 mouse myoblasts (passage number 10–15) were maintained in culture at 37°C, 5% CO_2 in air in a humidified atmosphere in C2C12 complete cell culture medium (DMEM supplemented with 10% (v/v) FCS, 2 mM L-glutamine, 100 units/mL penicillin, 0.1 mg/mL streptomycin, 0.25 μg/mL amphotericin B). Passaging and preparation of single cell suspensions for scaffold seeding was achieved by enzymatic digestion using a 0.25% (v/v) trypsin, 0.02% (w/v) EDTA solution in phosphate buffered saline pH 7.4 (PBS). Cell counts were assessed using Trypan blue exclusion and a haemocytometer; viable cell numbers were used to determine the final cell concentration for scaffold seeding.

Scaffolds (10 mm height × 5 mm diameter) were pre-wetted and sterilised with 70% (v/v) ethanol in dH_2O for 10 min followed by washing with PBS (pH 7.4) 3 times.

C2C12 cells were trypsinised, resuspended in C2C12 complete medium with 2% (v/v) FCS (C2C12 differentiation medium) and diluted to a final concentration of 5×10^5 cells in 50 μL. This aliquot was placed onto the scaffolds in a non-tissue culture treated 24-well plate and incubated at 37°C, 5% CO_2 in air for 4 h. Non-adherent cells were then removed and the scaffolds transferred to new plates. The constructs were cultured in C2C12 complete medium at 37°C, 5% CO_2 for 5 days.

Alkaline phosphatase activity

The differentiation of C2C12 myoblasts into the osteoblast lineage (under the influence of rhBMP-2 released from the microparticles) was visualized via alkaline phosphatase (ALP) activity [11]. To evaluate zonal release of rhBMP-2 from scaffolds, scaffolds were cut in half, seeded using C2C12 myoblast cells as described above and cultured for 5 days at 37°C, 5% CO_2 in air in a humidified incubator. Constructs were washed in PBS three times and fixed for 5 minutes with 95% (v/v) methanol in dH_2O. Samples were subsequently incubated in Napthol AS-MX buffer solution containing 1mg/ml Fast Violet B salt at room temperature for 30 min. A red colour substrate was precipitated on the cells by the action of cellular enzyme activity; this was observed using a Nikon dissection microscope (Model C-DSD230, Japan).

For quantitative measurement of alkaline phosphatase activity, a pNPP substrate system was used. In brief, constructs were fixed in 75% (v/v) ethanol and cellular extracts obtained by vigorous pipetting of 500 μl dH_2O containing 0.01% (v/v) TritonX detergent through the scaffold for 3 min. Cell extract was incubated with the pNPP substrate under alkaline conditions (60 min) and absorbance (λ 405) read on a F2 Microplate Fluorescence, Absorbance and Luminescence System (Labtech, UK). Values were compared against known pNP standards and matched to DNA content to compensate for changes in cell number. Data was expressed as μmol p-NP produced per minute per mg of DNA (as determined by the Hoechst DNA assay).

Hoechst 33258 DNA assay

Constructs were digested overnight in 1 mL papain solution (1.06 mg/mL in 0.1 M dibasic sodium phosphate, 0.005 M cysteine hydrochloride and 0.005 M EDTA pH 6.5) at 60°C. Aliquots of cell lysate (37.5 μl) were incubated with a 0.5 μg/ml (750 μl) solution of bisbenzamide in 0.01 M Trizma base, 0.01 EDTA, 0.1M NaCl pH 7 and 500 μl of

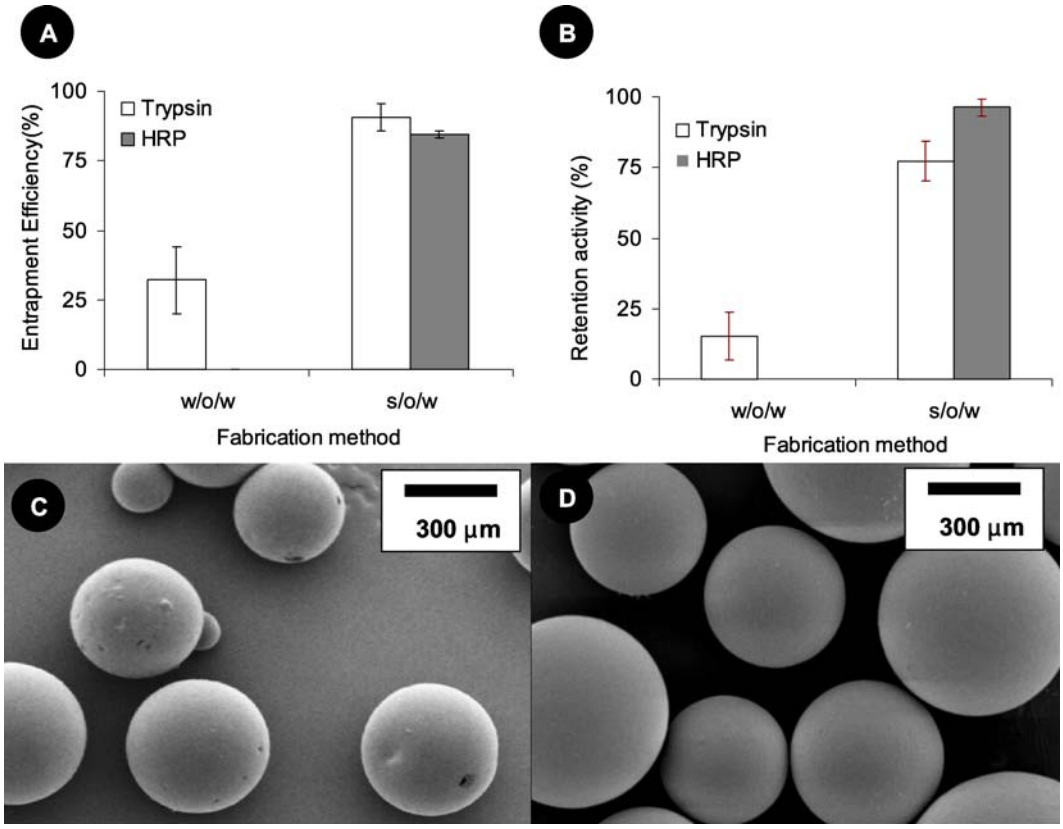

Fig. 1 Optimisation of protein-loaded microparticle fabrication: (A) Effect of microparticle fabrication method on entrapment efficiency. (B) Effect of microparticle fabrication method on enzyme activity. (C) SEM of w/o/w microparticles. (D) SEM of s/o/w microparticles

dilution buffer (0.01 M Trizma base, 0.01 EDTA, 0.1M NaCl pH 7) and the fluorescence measured immediately at excitation λ 360 nm and emission λ 460 nm on a F2 Microplate Fluorescence, Absorbance and Luminescence System (Labtech, UK). The amount of DNA within the scaffolds was determined using the standard curve of known concentrations of DNA (0–20 μg/ml; DNA from salmon testes) versus average fluorescence.

Results and discussion

Production of protein-loaded microparticles

Initial studies of microparticle production used the enzymes, trypsin and HRP, as model proteins whose activity, after release, could be quantified by a colorimetric assay. Conventional w/o/w emulsion methods for trypsin entrapment gave poor entrapment efficiencies of approximately 25% (Fig. 1a) and the measured enzyme activity fell to approximately 13% of the original trypsin solution used in microparticle manufacture (Fig. 1b). The enzyme activity value is not corrected for loss of protein and therefore the reduction in activity against control is explained by the escape of protein during manufacture (accounting for reduction to 25% activity) and the denaturing of the protein caused by the processing environment (accounting for the remaining reduction to 13%). It is therefore apparent that the double emulsion method is inefficient and reduces activity of the entrapped protein.

To address this issue we used a method described by Morita et al. [9] in which a solid dispersion of PEG and the protein is formed by freeze-drying an aqueous solution of the two components. The solid dispersion is then mixed with dichloromethane/P_{DL}LA and a single emulsion formed in water. In line with previous reports of this method, we measured entrapment efficiencies of above 75% for trypsin and similar values for retention of activity (Figs. 1a and b). This result was confirmed with the second model protein HRP (Figs. 1a and b). Both w/o/w and s/o/w fabrication methods generated morphologically similar microparticles of approximately 300 μm (Figs. 1c and d).

Formation of scaffolds by heat sintering

Scaffold formation by the sintering of microparticles requires extended exposure of the particles to temperatures in excess of the polymer Tg [10]. For future applications, the avoidance of heat inactivation of growth factors will be an important issue to address. Therefore, we studied the ability to use plasticizers to reduce the sintering temperature. We have studied 2 plasticizers, PEG (mw 400) and water.

Using DSC we measured the Tg of our P_{DL}LA material as a function of PEG 400 concentration. P_{DL}LA alone had a measured Tg of 47°C. Addition of 10%, 20% or 30% of PEG reduced the Tg of the blend to 42°C, 40°C and 38°C respectively. Scaffolds were formed by sintering each of the microparticle types for 4 hours at 70°C. The SEM images in Figs. 2 a,b,c and d show the internal structure of the resulting scaffolds. There is evidence that microparticles with higher PEG concentration deform to a greater extent during heating. This enhancement of fusion of neighbouring particles translates into enhanced compressive strength of the material (Fig. 2e).

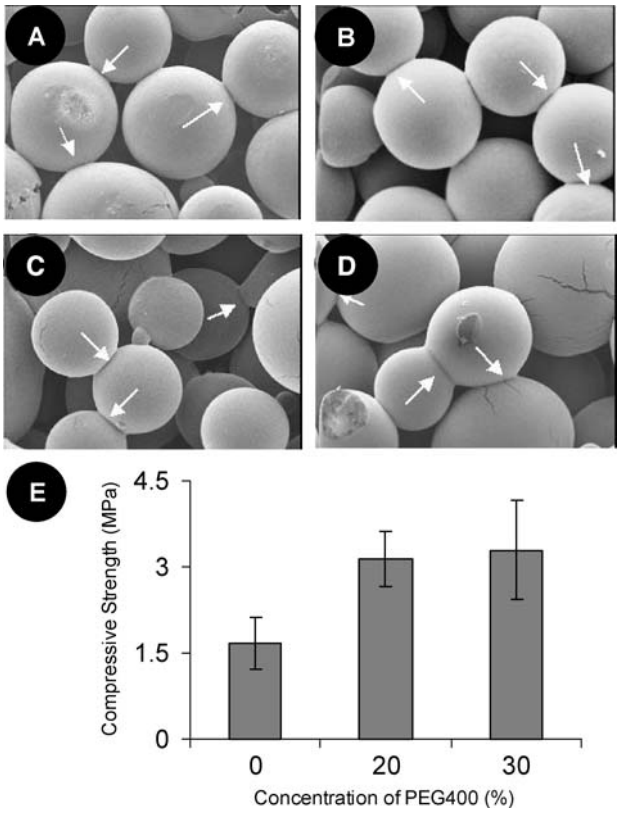

Fig. 2 Effect of PEG concentration on scaffold formation: (A to D) SEM images of scaffold internal morphology for 0%, 10%, 20% and 30% PEG 400 (arrows indicate fusion points between microparticles). (E) Influence of PEG concentration on scaffold compressive strength

Next we considered the additional role of water moisture on the PEG induced plasticization of the P_{DL}LA microparticles. Three batches of microparticles with measured water contents of 2.1%, 4.3% and 5.3% were sintered at 70°C. As shown in the SEM images in Figs. 3a, b and c increasing water content increased the mobility of the polymer and fusion of neighbouring particles. Clearly, the water content of the particles has the expected effect of further plasticizing the P_{DL}LA [12]. Figures 3d and e show that the increased fusion of particles increased compressive strength and lowered porosity. For subsequent studies we used fabrication conditions to minimise water content and keep the blend Tg high enough to avoid further polymer mobility under cell culture temperatures.

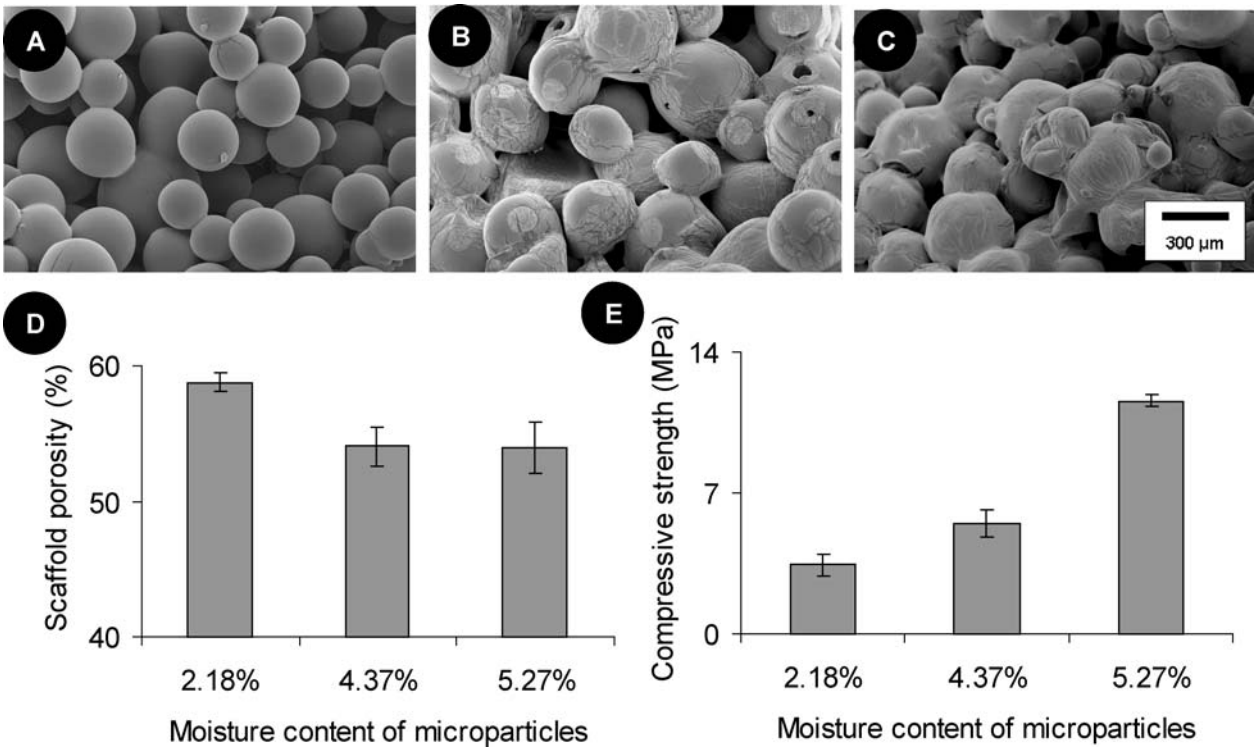

Fig. 3 Effect of water content on scaffold formation: (A to C) SEM images of scaffold structure with 2.18%, 4.37% and 5.27% water within P_{DL}LA/PEG (70:30) microparticles. (D) Scaffold porosity after sintering. (E) Compressive strength of scaffolds

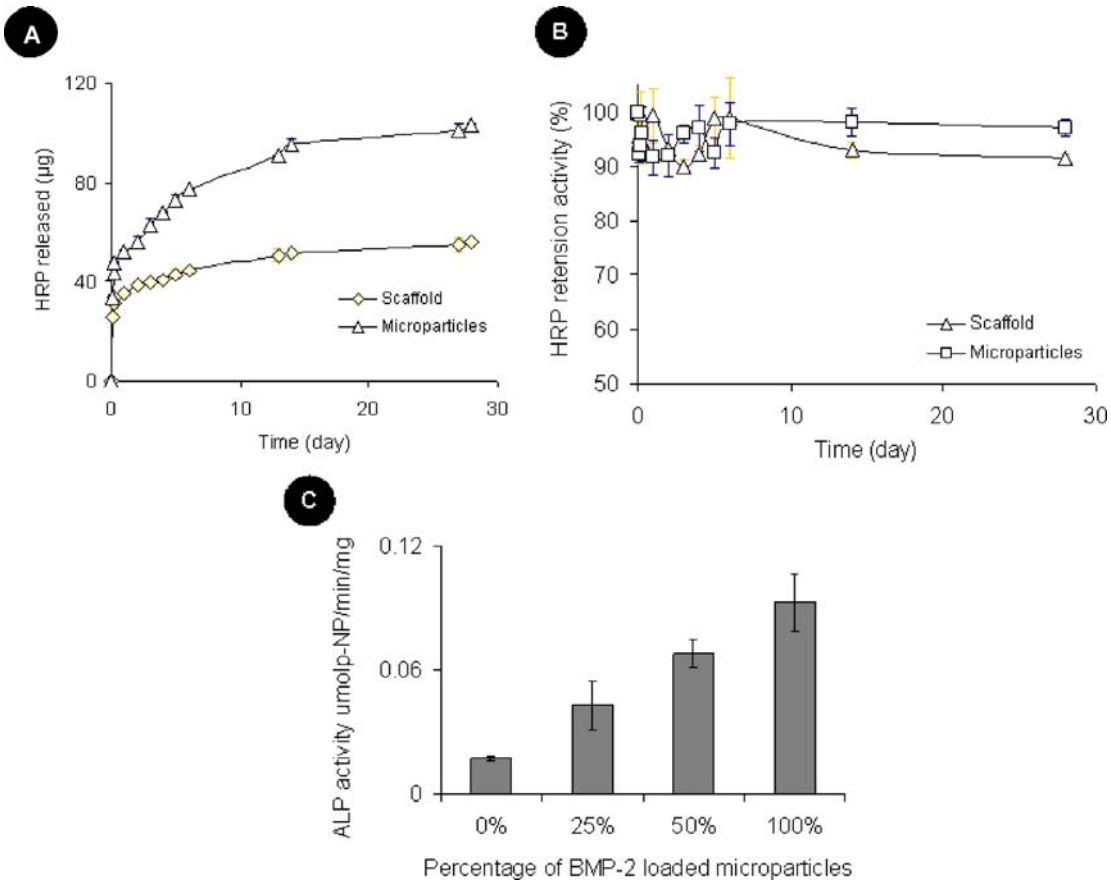

Fig. 4 Controlled release of model proteins and rhBMP-2 from scaffolds

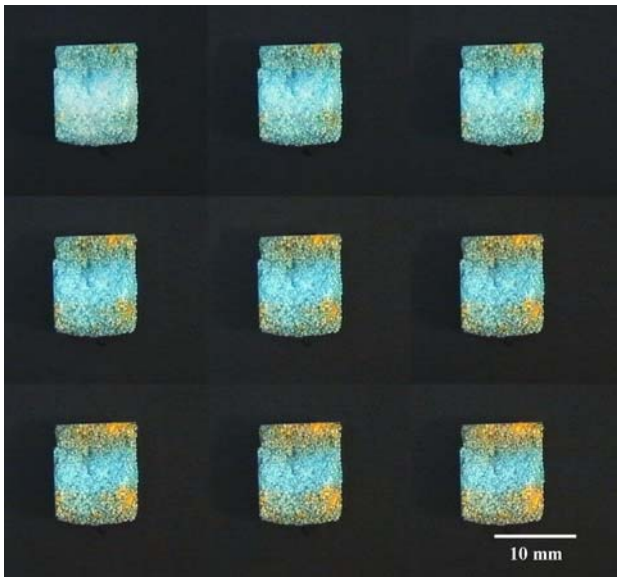

Fig. 5 Zonal release of horse radish peroxidase from trilayer scaffolds: Sequential images at approximately 2 minute intervals

Controlled release of model proteins and rhBMP-2 from scaffolds

Having established the conditions for scaffold fabrication, we studied the kinetics of release of proteins from the microparticle formulation. Figure 4a compares the release of HRP over a 30 day period from microparticles and microparticles fabricated into a scaffold. Controlled release over a 30 day period was achieved by both systems after an initial burst phase. Release from the scaffold was retarded compared to the free microparticles. Encouragingly, the activity of released protein was close to 100% of the control throughout the 30 day experiment period (Fig. 4b).

Next, we prepared rhBMP-2 loaded microparticles and prepared scaffolds by mixing rhBMP-2-loaded and rhBMP-2-free microparticles in varying ratios. C2C12 cells were cultured on the 3D scaffolds and the ALP activity was measured after 5 days of culture. The data in Fig. 4c confirms that rhBMP-2 released from these scaffolds promoted ALP expression in C2C12 cells. A linear relationship between the amount of rhBMP-2 loaded microparticles and ALP expression was found. This result indicates that mixing of growth factor loaded microparticles with blank microparticles is a simple method of tailoring the growth factor concentration without reformulating the microparticles.

Zonal release of HRP and RHBMP-2

Initial proof-of-principle studies on the zonal release of protein were performed using HRP-loaded particles. A tri-layer scaffold was formed with top and bottom layers composed

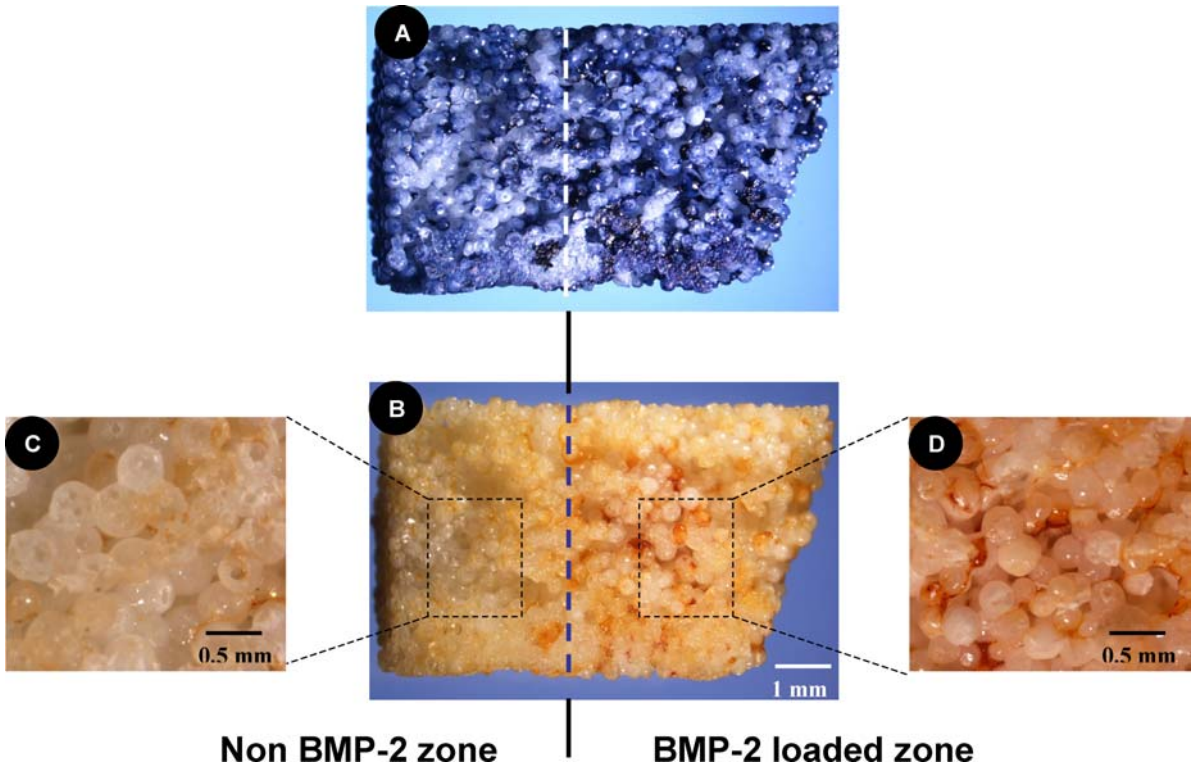

Fig. 6 Zonal release of rhBMP-2 from a bilayer scaffold seeded with C2C12: (A) Toluidine blue counterstain of the ALP stained scaffold shown in B. (B) Location of ALP staining demonstrating with the lack of C2C12 differentiation in the non-BMP loaded zone (C) and positive ALP staining (red) in the RHBMP-2 loaded zone (D)

of HRP-loaded particles and a middle layer of blank microparticles. Addition of water containing the HRP substrate TMB initiated release of HRP and enzyme activity resulted in the formation of a yellow product. The sequential images in Fig. 5 were taken at approximately 2 min intervals and show the expected zonal pattern to HRP activity. This study detected the protein released during the initial burst phase. However, given the controlled release demonstrated in Fig. 4a, the pattern of release would be expected to be maintained for many weeks.

Finally, we formed bi-layer scaffolds containing BMP-loaded microparticles in one layer and blank microparticles in the second layer. C2C12 cells were cultured for 5 days within these scaffolds and then induced ALP activity was visualised and compared against the location of toludine blue stained cells. In Fig. 6a, the toluidine blue stain confirms an even distribution of cells across the scaffold. Although the local distribution of cells was patchy, both zones of the scaffold contained approximately equal cell numbers. ALP activity was concentrated within the rhBMP-2 loaded microparticle zone (Figs. 6 b, c and d). The effect of the rhBMP-2 was not exclusive to the zone composed of rhBMP-2 loaded microparticles as some diffusion of the molecule occurred within the scaffold.

Conclusions

Zonal release of proteins with $P_{DL}LA$ scaffolds has been demonstrated. To develop this scaffold technology it has been necessary to refine a solid-in-oil-in-water method of microparticle production to achieve high entrapment efficiencies and the release of active protein. Doses of rhBMP-2 within scaffolds have been tuned by the facile method of varying ratios of microparticles containing rhBMP-2 and polymer-only microparticles. Zonal activity of rhBMP-2 has been achieved.

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